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THE ANTISEPTIC POTENTIAL OF THE FORMULATED LIQUID SOAP FROM THE METHANOLIC EXTRACT OF *Sepioteuthis lessoniana* (Bigfin Reef Squid) INK AGAINST *Staphylococcus aureus* ATCC 25923

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Abstract: The majority of hospital-related infections worldwide are related to skin contact. In this study, the antiseptic potential of liquid soap from the methanolic extract of *Sepioteuthis lessoniana* ink against *Staphylococcus aureus* was determined. A non-randomized, controlled trial was done with positive control of Safeguard Liquid Hand Soap. The different concentrations (0.5% v/v, 1% v/v, and 5% v/v) of *Sepioteuthis lessoniana* ink extract soap were tested for their inhibitory activity. The Durham Tube Test was utilized to determine the antiseptic effect of the various concentrations, the minimum time for antiseptic effect to occur, and the corresponding efficacy of the commercial disinfecting agent. The study showed that all three experimental groups and the positive group inhibited the growth of the bacteria in as short as 5 minutes from contact with the bacteria. Safeguard Liquid Hand Soap which contains the active ingredient 0.5% chloroxylenol and the lowest formulation of 0.5% v/v concentration of *Sepioteuthis lessoniana* ink extract liquid soap exhibited similar results during the experiment. Therefore, formulated soap from the ink extract of *Sepioteuthis lessoniana* demonstrated antiseptic properties that are comparable to that of Safeguard Liquid Hand Soap.

Keywords: Antiseptic activity, contact time, *Sepioteuthis lessoniana* ink extract, *Staphylococcus aureus*

I. INTRODUCTION

The number of skin illnesses brought about by *Staphylococcus aureus* has increased drastically. Primarily, these result from poor hygienic practices, such as exposed burns, scrapes, minor cuts, and wounds at surgical sites. *Staphylococcus aureus* may cause an increased risk of COVID-19 bacterial coinfections leading to acute pneumonia, or more severe COVID-19

symptoms when it infects the patient's lungs (Chandran et al., 2021).

Staphylococcus aureus is a species of bacteria that is typically present on human skin and mainly causes minor skin infections. However, when the bacteria penetrates a person's skin more deeply and affects other organs, it can become fatal (Kwiecinski & Horswill, 2020). Those infected with both SARS-CoV-2 and *S. aureus* succumb at a rate of 61.7%, which indicates that the mortality rate significantly increases compared to patients who were only infected with SARS-CoV-2 (Adalbert et al., 2021).

There is a pressing need for novel antiseptics due to antimicrobial resistance that has been brought about through the negligent usage of antimicrobials (Anvarsha & Kalyani, 2021). Today, drug-resistant infections are a serious threat to people's health and discovering new antibiotics is one of the means to address this public health dilemma.

The tropical Indo-Pacific is heavily populated by the neritic squid *Sepioteuthis lessoniana.* This species is valued for human consumption as food and is of major importance to Southeast Asian fisheries (Runck, 2021). When these animals hunt and detect the presence of other animals, it releases from their ink sac a substance which they use as a protective mechanism to block the predators' view, enabling them to flee. The ink contains various substances, such as melanin, enzymes, polysaccharides, catecholamines, metals like cadmium, lead, and copper, as well as amino acids like glutamate, taurine, alanine, leucine, and aspartic acid. Moreover, in many studies, the ink has been shown to have an anticarcinogenic, antioxidant, antihypertensive, immunomodulation, and antibacterial activity.

Biodiversity may be seriously threatened by the rising amount of waste products created naturally by animals, plants, as well as humans. *S. lessoniana* ink is one such waste product from the seafood processing industry that researchers may look into which may ultimately benefit our society (Binsi & Zynudheen, 2019).

Ramasamy et al. (2011) reported that the methanolic extract of *S. lessoniana* had varying outcomes in different concentrations against *S. aureus*. Their study showed a weak activity with a zone of inhibition that measured 7 to 10 millimeters in diameter at 50% concentration, a good activity with a zone of inhibition that measured 11 to 15 millimeters in diameter at 75% concentration, and a very good activity at 100% concentration with a zone of inhibition that measured above 16 millimeters in diameter (Ramasamy et al., 2011).

The primary objective of this study is to transform a waste product from *Sepioteuthis lessioniana* into a pharmaceutical product against a deadly pathogen during this time of crisis. This innovation introduces the versatility of drug-making through maximizing waste products and becoming interdependent with inactive substances.

II. METHODOLOGY

Samples of the juvenile stage of *Sepioteuthis lessoniana*, each measuring 19 to 33 millimeters in length, collected from the fishing spot of Sitio San Roque II, Barangay Ipil, Ormoc City, were certified and verified by the Department of Biology, School of Arts and Sciences, University of San Carlos, Cebu City, Cebu, Philippines, The samples were stored in an ice bucket for transport after being preserved in slightly acidic electrolyzed water ice (SAEW-ice) in a clean amber jar.

The Cebu Doctors' University (CDU) Institutional Biosafety Committee certified the purity of the *Staphylococcus aureus* ATCC 25923 specimen obtained from Scientific Biotech Specialty, Inc. Durham's Fermentation Tube test was employed to evaluate the presence of the growth of

Staphylococcus aureus ATCC 25923 after 24 hours of incubation.

Before the ink was extracted, the *Sepioteuthis lessoniana* samples were thoroughly cleaned with distilled water. The ink was released into a 500 mL beaker by gently piercing the ink sac with a knife. Three hundred milliliters of ink were placed in a freezer capable of reaching a temperature of -18°C and left there for 3 hours without being opened. The frozen ink was then dried for three hours in a hot air oven to remove any remaining water. Using a mortar and pestle, the dried ink was pulverized into a fine powder. A total of 30 grams of ink powder was collected and mixed with 90 mL of methanol in a sterile 120 mL glass amber bottle by parallel extraction. The preparation was refrigerated at 4°C for 72 hours, placed on a laboratory shaker at room temperature for 8 hours, and centrifuged to separate the supernatant liquid. The supernatant was subjected to UV light for about 2 hours to ensure its sterility, and purified using a rotary evaporator at 40°C for 25 minutes at 150 rpm. Ten grams of the purified methanolic ink extract was mixed with 10 mL of distilled water to make a stock solution for further analysis.

The presence of melanin in the ink extract was tested using the Xanthoproteic and Millon's tests. During the Xanthoproteic test, a few drops of the stock solution were added to 1 mL of concentrated nitric acid. The solution was mixed and heated, then the solution was cooled with tap water and 1 mL of 40% sodium hydroxide was added to make the solution alkaline. Orange coloration of the solution indicates the presence of aromatic amino acids (Basnet, 2020). To prove the presence of melanin, Millon's test was performed. To 1 mL of the stock solution in a test tube, a few drops of Millon's reagent were added. The test tube was boiled gently for 30 seconds. A crimson or red precipitate indicates the presence of melanin (Karki, 2018).

The presence of terpene was detected using the Liebermann-Burchard and Salkowski tests. Following the Liebermann-Burchard test procedure, 1 mL of chloroform, 2-3 mL of acetic anhydride, and 1-2 drops of concentrated sulfuric acid were added to 1 mL of the stock solution in a test tube. A positive result is shown by a red discoloration of the solution (Jeyasanta & Patterson, 2020). Moreover, the Salkowski test procedure was performed by mixing 1 mL of the stock solution and 2 mL of chloroform. Three mL of concentrated sulfuric acid were added carefully to the mixture and a reddish-brown coloration confirms the presence of terpene (Das et al., 2014).

The presence of phenolic compounds was detected using the Ferric Chloride and the Bromine Water tests. The Ferric Chloride test was done by adding a neutral solution of ferric chloride drop by drop to 1 mL of the stock solution in a test tube. A greenish discoloration of the solution confirms the presence of phenols (Singh, 2023). The Bromine Water test was performed by mixing 1 mL of the stock solution with 1 mL of glacial acetic acid, and adding bromine water drop by drop to the mixture. The disappearance of the yellow bromine color indicates the presence of phenolic compounds (Bhagya, 2018).

The hot process method was utilized to formulate the liquid hand soap. Seven hundred milliliters of coconut oil were heated to 60°C for 20-30 minutes. For the lye-water solution, 175 g of potassium hydroxide were mixed into 584.5 mL of distilled water. The solution was stirred until the potassium hydroxide dissolved. The lye-water solution was poured slowly into the heated coconut oil and stirred carefully. The lye-water solution and heated coconut oil mixture were added to the glycerin. The resulting solution was heated to a temperature of 70°C for 3-4 hours. A sufficient amount of distilled water was added to prepare 1000 mL of soap. Ten grams of Sodium Borate powder was added

to the liquid soap to neutralize and preserve it for further testing.

Three pure ink extract stock solutions, namely, 2.08%, 4.17%, and 20.8%, were prepared, by mixing 0.63 mL, 1.26 mL, and 6.30 mL, respectively, of the pure ink extract with enough distilled water to make 30 mL of each of the stock solutions.

Three different liquid soap concentrations were formulated to be used as the experimental treatment groups. Experimental treatment group 1, labelled 0.5% v/v soap, was formulated by mixing 30 mL of the 2.08% v/v stock solution with 95 mL soap base. Experimental treatment group 2, labelled 1% v/v soap, was formulated by mixing 30 mL of the 4.17% v/v stock solution with 95 mL soap base. Experimental treatment group 3, labelled 5% v/v soap, was formulated by mixing 30 mL of the 20.8% v/v stock solution with 95 mL soap base. The positive control treatment group used 125 mL Safeguard Liquid Hand Soap. The formulated liquid hand soaps were stored at room temperature in a clean container.

The purchased Culti-Loops™ of *Staphylococcus aureus* ATCC 25923 were subcultured in blood agar medium through the direct streak method with the assistance of a registered medical technologist. A total of 3 plates were streaked using the same loop. The plates were incubated at 37°C for 24 hours. A stock inoculum was prepared by inoculating a bacterial colony from one of the culture media onto 10 mL of NSS in a test tube.

The Durham Tube Test was used to assess the relative effectiveness of the extract against the bacterial culture and the minimum time to produce its antiseptic effect. Five test tubes were prepared, labelled Soap, 5 minutes, 10 minutes, 15 minutes, and 30 minutes, for each of the treatment groups (0.5%, 1%, and 5% of the ink extract) as well as for the control treatment group for a total of 20 test tubes. Five milliliters of each of the three soap concentrations and of the Safeguard Liquid Hand Soap were placed in the respective test tubes labelled Soap. Five milliliters of lactose broth were placed in each of the other test tubes with the Durham tubes. The timer was started as soon as an inoculating loop from the stock inoculum was transferred to each of the tubes labelled Soap. After 5 minutes, 10 minutes, 15 minutes, and 30 minutes, one mL of the soap and inoculum mixture was transferred to the respective lactose broth tubes containing Durham tubes. Two trials were done for this experiment. Turbidity and the presence of gas bubbles in Durham tubes after 24 hours of incubation are signs of bacterial growth (Hartline, 2023).

III. RESULTS AND DISCUSSION

Table 1: Distribution of Time of Bacterial Inhibition Across Groups

Treatment Group	Inhibition Time	
	Trial 1	Trial 2
Safeguard Liquid Hand Soap	5 minutes	5 minutes
0.5% v/v *Sepioteuthis lessoniana* ink extract soap	5 minutes	5 minutes
1% v/v *Sepioteuthis lessoniana* ink extract soap	5 minutes	5 minutes

Table 1 shows that after only 5 minutes of contact time of the Safeguard Liquid Hand Soap and the three ink extract soap concentrations (0.5%, 1%, and 5% v/v) of *Sepioteuthis lessoniana* with the inoculum of *Staphylococcus aureus* ATCC 25923, bacterial growth was inhibited.

The findings in the experimental groups after 5 minutes, 10 minutes, 15 minutes, and 30 minutes of contact time revealed no indication of bacterial growth. For trials 1 and 2, this was shown as a transparent, homogeneous mixture without any sign of gas formation in the Durham tubes.

The active ingredient of the Safeguard Liquid Hand Soap is 0.5% chloroxylenol and is branded as a phenol antiseptic that acts by disrupting the bacterial cell wall and inactivating intracellular enzymes, rendering the cell inactive (Bednarek et al., 2023). The coagulated protein and nucleic acid content of the bacterial cells causes fast cell death at extremely high chloroxylenol doses (National Institutes of Health, 2023). Safeguard was found to have the highest efficacy compared to other commercial liquid hand soaps such as Johnson and Johnson, Dettol, Lifebuoy, and Lux in terms of its minimum inhibitory concentration against *Staphylococcus aureus* and *Eschericia coli* (Sajed et al., 2014).

The Durham tubes did not float and showed no signs of gas formation, indicating no bacterial growth on test tubes after only 5 minutes of contact time in the three experimental treatment groups displaying a clear and homogeneous mixture in both trials after 24 hours of incubation. The experimental treatment groups showed no bacterial growth at 10 minutes, 15 minutes, and 30 minutes. Due to the bioactive components of the ink extract present, the treatment groups were able to stop the bacterial growth after just five minutes of contact.

The squid ink contains chemicals that have antibacterial action against various microorganisms and could be used as a natural antibiotic (Jismi et al., 2018). The crude ink extract of *Sepioteuthis lessoniana* showed good antibacterial activity against *Staphylococcus aureus* (Hossain et al., 2019). It has a strong antimicrobial activity against microorganisms that form biofilms, especially after heat treatment (Nicomrat & Tharajak, 2015). Its methanolic extract has shown varying results from previous studies at different concentrations against *Staphylococcus aureus*, with a 7-10 mm diameter zone of inhibition formed at 50% concentration, 11-15 mm diameter zone of inhibition at 75% concentration, and greater than 16 mm diameter zone of inhibition at 100% concentration (Ramasamy et al., 2011). The antibacterial properties of squid ink were reported to be effective against some pathogens examined, including *Staphylococcus aureus*. The antibacterial efficacy of 200 µL of squid ink extract as a zone of inhibition against *Staphylococcus aureus* was remarkable at 24 mm (Nadarajah et al., 2017).

A chemical with high potency can elicit a specific response at low doses, whereas a substance with low potency can only do so at larger quantities (Whalen et al., 2015). The results, therefore, demonstrate that the experimental groups had an antiseptic potential as there were no signs of bacterial growth as indicated by absence of gas formation in the Durham tubes. Hence, the 0.5% v/v soap concentration is the most potent out of all the formulated experimental treatment groups.

IV. CONCLUSION

There is no significant difference in the inhibition of *Staphylococcus aureus* ATCC 25923 between the positive group's contact time and the various concentrations of *Sepioteuthis lessoniana* ink extract soap. Therefore, all the methanolic squid ink extract liquid soap concentrations produced an antiseptic potential against the *Staphylococcus aureus* ATCC 25923. The researchers recommend developing an optimum formulation that integrates the features of a standard liquid soap in terms of physicochemical stabilities, consistency, odor, color, and aesthetics. On top of that, the researchers would recommend experimenting with soap concentrations

lower than 0.5% v/v, alter contact times, identify the ink extract's mechanism of action against pathogens, and to identify the safety profile of the ink extract.

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